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Fungal gene expression during ectomycorrhiza formation

F. Martin, P. Laurent, D. de Carvalho, T. Burgess, P. Murphy, U. Nehls, and D. Tagu

Abstract: Ectomycorrhiza development involves the differentiation of structurally specialized fungal tissues (e.g., mantle and Hartig net) and an interface between symbionts. Polypeptides presenting a preferential, up-, or down-regulated synthesis have been characterized in several developing ectomycorrhizal associations. Their spatial and temporal expressions have been characterized by cell fractionation, two-dimensional polyacrylamide gel electrophoresis, and immunochemical assays in the *Eucalyptus* spp. – *Pisolithus tinctorius* mycorrhizas. These studies have emphasized the importance of fungal cell wall polypeptides during the early stages of the ectomycorrhizal interaction. The increased synthesis of 30- to 32-kDa acidic polypeptides, together with the decreased accumulation of a prominent 95-kDa mannoprotein provided evidence for major alterations of *Pisolithus tinctorius* cell walls during mycorrhiza formation. Differential cDNA library screening and shotgun cDNA sequencing were used to clone symbiosis-regulated fungal genes. Several abundant transcripts showed a significant amino acid sequence similarity to a family of secreted morphogenetic fungal proteins, the so-called hydrophobins. In *P. tinctorius*, the content of hydrophobin transcripts is high in aerial hyphae and during the ectomycorrhizal sheath formation. Alteration of cell walls and the extracellular matrix is therefore a key event in the ectomycorrhiza development. An understanding of the molecular mechanisms that underlies the temporal and spatial control of genes and proteins involved in the development of the symbiotic interface is now within reach, as more sophisticated techniques of molecular and genetic analysis are applied to the mycorrhizal interactions.

Key words: cell walls, ectomycorrhiza, ectomycorrhizins, fungal development, hydrophobins, symbiosis-regulated polypeptides.

Résumé : Le développement de la symbiose ectomycorhizienne implique la différenciation de structures multicellulaires fongiques (le manteau et le réseau de Hartig) et la formation d'une interface spécialisée entre symbiotes. Des polypeptides spécifiques de la symbiose (ectomycorhizines) et des polypeptides dont la synthèse est modifiée par le développement de l'ectomycorhize ont été mis en évidence dans plusieurs associations symbiotiques. L'expression spatio-temporelle de ces polypeptides régulés par la symbiose a été caractérisée par fractionnement cellulaire, électrophorèse bidimensionnelle, et immunoquantification des protéines dans l'ectomycorhize d'*Eucalyptus* spp. – *Pisolithus tinctorius*. Ces études ont montré l'impact des premières étapes de l'interaction mycorhizienne sur la synthèse des polypeptides pariétaux du mycélium. Les modifications de la paroi fongique du basidiomycète *Pisolithus tinctorius* se manifestent par une stimulation de la synthèse de polypeptides acides de 30–32 kDa et, simultanément, une chute de la biosynthèse d'une mannoprotéine majeure de 95 kDa. Des gènes fongiques régulés par le développement de la symbiose ont été clonés par hybridation différentielle et séquençage systématique d'ADNc. Plusieurs de ces ADNc codent pour des protéines fongiques similaires aux hydrophobines. Ces protéines sont sécrétées dans la paroi et le milieu extracellulaire et sont impliquées dans de nombreux processus morphogénétiques. Chez *P. tinctorius*, les transcrits codant pour les hydrophobines sont élevés dans les hyphes aériens et lors de la formation du manteau fongique. Les modifications de la paroi fongique et de la matrice extracellulaire apparaissent donc comme une étape essentielle du développement symbiotique. Les approches moléculaire et génétique devraient permettre une meilleure compréhension de l'expression de ces gènes et protéines impliqués dans la formation de l'interface symbiotique.

Mots clés : parois, ectomycorhize, ectomycorhizines, développement fongique, hydrophobines, polypeptides régulés par la symbiose.

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Table 1. Time frame of the formation of ectomycorrhiza of *Eucalyptus globulus* – *Pisolithus tinctorius* 441 obtained in Petri dish.

Time	Developmental stage	Anatomical features
0–12 h	Preinfection	Hyphal contacts with the root
12–24 h	Symbiosis initiation	Fungal attachment to the epidermis
24–48 h	Fungal colonization	Initial layers of mantle Hyphal penetration between epidermal cells
48–96 h	Symbiosis differentiation	Rapid buildup of mantle hyphae Hartig net proliferation
96 h – 7 days	Symbiosis function	Mantle well developed and tightly appressed to epidermal cells End of Hartig net growth

Introduction

The interaction between certain compatible soil fungi, mainly basidiomycetes, and trees results in the development of a complex root organ where the two organisms no longer function independently, but rather as a new symbiotic structure called an ectomycorrhiza (Harley and Smith 1983). The process of root colonization by the ectomycorrhizal fungus, the differentiation of specialized multicellular structures, and the establishment of an active ectomycorrhiza require a complex sequence of interactions between fungal and root cells. The mature organization of the ectomycorrhizal symbiosis varies with the host and fungal species involved. However, early stages of ectomycorrhiza development present well-characterized similar morphological transitions: (*i*) contact of the mycelium with the surface of the root apical zone; (*ii*) growth onto the exposed surface; (*iii*) adhesion to roots; (*iv*) penetration between epidermal cells and cortical cells; and (*v*) formation of the fungal mantle and intercellular Hartig net with concomitant coordinated alteration in the root structure (Kottke and Oberwinkler 1986, 1987; Horan et al. 1988; Massicotte et al. 1987, 1989; Dexheimer and Pargney 1991; Bonfante and Perotto 1992). Colonization of the root by mycorrhizal hyphae and morphogenesis of the symbiotic structures induce the preferential expression of a particular set of host proteins, some of which are thought to be involved in defense reactions (Hilbert and Martin 1988; Hilbert et al. 1991; Martin and Hilbert 1991; Simoneau et al. 1993; Burgess et al. 1995; Albrecht et al. 1994). On the other hand, penetration and colonization of the host tissues are accompanied by major shifts in gene expression in the fungal symbiont (Tagu et al. 1993; Martin et al. 1995). The transcripts of in planta regulated fungal genes are likely involved in the development and maintenance of a compatible ectomycorrhizal interaction (Martin and Tagu 1994).

Current approaches to identify factors that are involved in the signaling between symbionts and development of the symbiotic structures involve molecular cloning of genes and purification of proteins that present induced, up- or down-expression during the early stages of ectomycorrhiza development. Identification of the symbiosis-regulated (SR) genes and polypeptides might elucidate some of the cellular and molecular events involved in mycorrhiza interactions. This review summarizes what is known or can be speculated about fungal gene expression during the early stages of ectomycorrhiza development.

Alteration of fungal protein biosynthesis

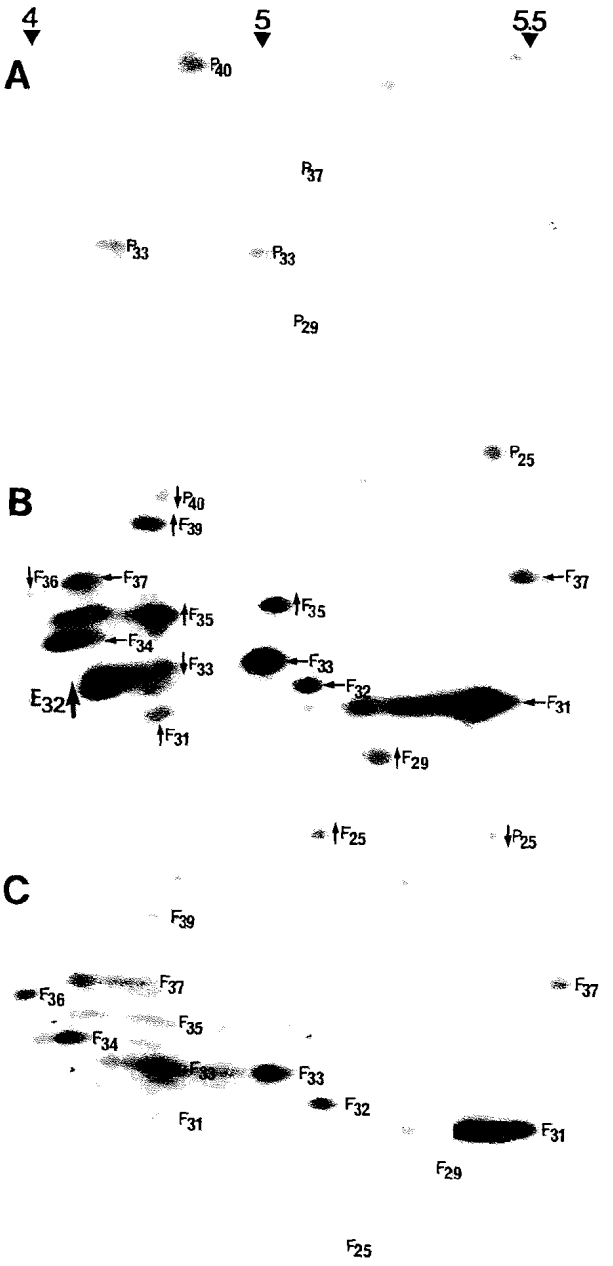
To characterize fungal and root proteins whose expression is regulated during penetration and colonization of *Eucalyptus globulus* roots by the basidiomycete *Pisolithus tinctorius* (Table 1), changes in protein composition between the individual partners and fully developed ectomycorrhizas have been investigated by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) (Hilbert and Martin 1988). Mycorrhiza formation was accompanied by a differential accumulation of polypeptides. These alterations in protein content fell into three distinct categories: polypeptides having a lower concentration in symbiotic tissues, polypeptides showing an enhanced accumulation, and symbiosis-specific polypeptides, referred to as ectomycorrhizins (Hilbert and Martin 1988). These results were further supported by temporal examination of protein synthesis by in vivo labelling with [^{35}S]methionine in early stages of mycorrhizal development (Hilbert et al. 1991; Burgess et al. 1995) (Fig. 1). Simoneau et al. (1993, 1994), examining the symbiosis between *Betula pendula* and *Paxillus involutus*, also showed that SR polypeptides (i.e., up- or down-regulated expression), including ectomycorrhizins (i.e., symbiosis specific), accumulated during early mycorrhiza development. Occurrence of ectomycorrhizins in ectomycorrhiza has been challenged by Guttenberger and Hampp (1992).

The rate of biosynthesis and accumulation of upregulated polypeptides and ectomycorrhizins in planta are strongly affected by the aggressiveness (i.e., rate of ectomycorrhiza development) of a fungal isolate. SR polypeptides from the most aggressive isolates of *P. tinctorius* accumulated in mycorrhizal roots at a higher rate than those from the less aggressive isolates (Burgess et al. 1995).

Developmental regulation of cell wall proteins

Cytological investigations have stressed that the mechanisms of root surface recognition and binding of the hyphal tip are key features of endo- and ecto-mycorrhizal symbioses (Piché et al. 1988; Bonfante 1988; Bonfante and Perotto 1992). It is likely that mycorrhizal fungi, as most biotrophic fungi, have developed a large set of mechanisms by which they mediate binding to the host root. Evidence is accumulating that they secrete specific binding glycoproteins able to recognize and bind to specific protein or polysaccharide structures present on the root surfaces (Piché et al. 1988; Lapeyrie et al. 1989;

Fig. 1. Changes in protein biosynthesis during development of *Eucalyptus grandis* ectomycorrhiza with isolate H2144 of *Pisolithus*. Fluorograms of 2D PAGE areas highlighting a cluster of fungal acidic polypeptides having an enhanced synthesis. (A) Non-inoculated plant roots. (B) Mycorrhizal roots. (C) Free-living mycelium. Similar amounts of radioactivity have been loaded onto the gels. Reference plant (P) and fungal (F) polypeptides are indicated to aid orientation. Comparison of mycorrhizal roots, 4 days after contact, with the free-living partners revealed polypeptides having unaffected (→), enhanced (↑), and decreased (↓) synthesis and the ectomycorrhizin E_{32} (↑). Subscripts denote molecular mass (kDa), and superscripts stand for apparent pI of the polypeptides. pI values are shown at the top of the figure. (After Burgess et al. 1995.)

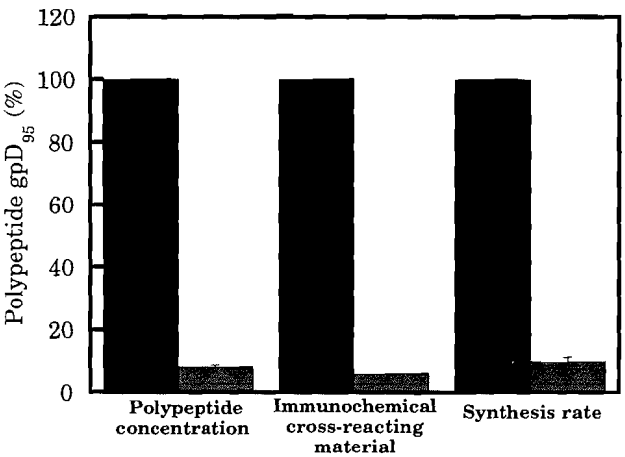


Lei et al. 1990, 1991; Bonfante and Perotto 1992). It has been suggested (Martin et al. 1995) that these secreted fungal proteins would be capable of mediating a specific, high-affinity interaction between the host and the mycobiont.

The composition of the symbiotic interface of ectomycorrhizas has been studied using cytochemistry to detect and locate specific sugars. This revealed differences in cell wall composition between nonmycorrhizal and mycorrhizal tissues (Paris et al. 1993). Fungal adhesion is presumably mediated by a mucilaginous matrix covering the hyphae and the root surface (Bonfante 1988; Piché et al. 1988; Lapeyrie et al. 1989). Microscopic investigations showed that orientated fimbriae containing Concanavalin A recognized glycoproteins are involved in the attachment (Lei et al. 1991). The secretion of these extracellular glycoproteins appears to be a key step in the subsequent colonization of the root surface by hyphae in compatible associations. A layer of extracellular fibrillar polymers is present in the extracellular matrix of the free-living mycelium of *Laccaria bicolor* (Lei et al. 1991) and *P. tinctorius* (Lei et al. 1990) even before the interaction with the root. At the contact sites between hyphae and root cells an increased secretion of these extracellular fibrillar polymers takes place. It is likely that a concomitant reorganization of the extracellular fibrillar polymers occurs, observed on microscopic sections as an accumulation and orientation of the extracellular polymeric fimbriae towards the host cell. In contrast, in the noncompatible interactions no fibrillar material was secreted, and mycorrhiza development was delayed.

A cluster of upregulated acidic polypeptides (AP) accumulating in eucalypt ectomycorrhizas (Fig. 1) has become the subject of intense investigations. In inoculated roots, several 30- to 32-kDa APs are synthesized at a developmental stage when other major fungal polypeptides are barely detected on the fluorogram of mycorrhiza-labelled proteins, suggesting that they are of plant origin or are fungal polypeptides secreted at a high rate onto the root surface (Hilbert et al. 1991; Burgess et al. 1995). 2D PAGE analysis and peptide mapping suggest that the early ectomycorrhizins (E_{32a}, E_{32b}) initially characterized in *E. globulus* mycorrhizas (Hilbert et al. 1991) and then in *Eucalyptus grandis* mycorrhizas (Burgess et al. 1995) belong to this family of fungal developmentally regulated APs. Further investigations showed that 30- to 32-kDa APs are polypeptides secreted by *P. tinctorius*. They are either located in cell walls or abundantly secreted in the extracellular medium by the free-living mycelium (D. De Carvalho, P. Laurent, and F. Martin, unpublished data). Some isoforms are constitutively expressed in the nonsymbiotic mycelium, whereas other isoforms are preferentially expressed in the fungal symbiotic tissues (Fig. 1). Many, if not all, proteins secreted by fungi are glycosylated (Peberdy 1994). However, the secreted APs of *P. tinctorius* are not recognized by mannose-specific lectins. Several 30- to 32-kDa APs have been purified by 2D PAGE and characterized by peptide mapping using V8 protease. Peptide digests of the isolated isoforms are similar suggesting that the occurrence of various isoforms is the result of post-translational modifications. As part of our efforts to understand the role of these secreted fungal polypeptides, a 30-kDa isoform of these symbiosis-regulated acidic polypeptides (SRAPs) has been purified to homogeneity and is currently sequenced.

Fig. 2. Developmental downregulation of the cell wall mannoprotein gpD_{95} from *Pisolithus tinctorius* in eucalypt mycorrhiza. The amount and synthesis rate of the mannoprotein were estimated in cell walls of free-living mycelium of *Pisolithus tinctorius* 441 (■) and 7-day-old ectomycorrhiza of *P. tinctorius* – *Eucalyptus globulus* (▩). The gpD_{95} concentration was estimated by densitometry of silver-stained gels and Western blotting analysis using anti-gpD_{95} antibodies. The synthesis rate was estimated by densitometric quantition of [^{35}S]gpD_{95} after in vivo labelling of cell wall proteins. Data are the mean of three replicates (± SD). Sample loading has been normalized using the content of fungal ergosterol and chitin as an estimate of the fungal material within the symbiotic tissues (P. Laurent, D. De Carvalho, and F. Martin, unpublished data).



How can we envisage a role of secreted proteins in mycorrhiza development? As stressed above, to complete the differentiation of symbiotic tissues, fungal cells have to fulfill at least two requirements: attach to the root surface and adhere tightly to form the mantle. The accumulation of fungal SRAPs coincides with the formation of fungal fimbriae bridging the colonizing hyphae and the eucalypt root surface (Lei et al. 1991). We have therefore suggested that SRAPs may correspond to some of the fimbriae proteins (Martin et al. 1995). In any case, the preferential expression of specific secreted proteins in the matrix of the symbiotic interface suggests that, as expected, this is a key region in the plant–fungal interaction.

Thus, the properties of cell walls may be adjusted through the alteration in their composition. To investigate further the changes in cell wall properties, we have characterized the protein content of fungal cell walls in the symbiotic tissues. For example, the most abundant cell wall protein in the cell wall of the free-living *P. tinctorius*, an acidic mannoprotein, is only present in low concentration in the fungal tissues of ectomycorrhizal roots (Fig. 2). Based on these results, this 95-kDa glycoprotein, which we designate gpD_{95}, was further characterized. For this purpose, the gpD_{95} protein was purified by 2D PAGE, and this preparation was used to raise a rabbit antiserum. The anti-gpD_{95} serum reacted strongly with a single protein band of 95 kDa on a gel blot of proteins from free-living mycelium. This was a remarkable finding since affinity blotting with concanavalin A (ConA) and the *Gladiolus nivalis* agglutinin (GNA) had shown that gpD_{95} is glycosylated and is characterized by terminal mannose chains. This feature implied that the antiserum raised against the gpD_{95} glycoprotein would likely cross-react with other glycosylated cell wall proteins. Apparently, either the glycosyl side chains containing terminal mannose residues are not very antigenic or they are unique for the gpD_{95} protein. Time course experiments using anti-gpD_{95} serum have shown that gpD_{95} is downregulated at a very early stage of mycorrhiza formation (P. Laurent and F. Martin, unpublished data).

The increased synthesis of 30- to 32-kDa APs and the downregulation of gpD_{95} provide further evidence for molecular differentiation of the fungal cell wall and extracellular matrix during the early stages of the ectomycorrhizal interaction. Polysaccharide (Bonfante and Perotto 1992) and protein (our studies) contents of fungal cell walls are altered during the differentiation of the symbiotic interface. This is in agreement with the generally held contention that the extracellular matrix and its associated secreted proteins play an important part in fungal development (Wessels 1992, 1993; Peberdy 1994; Templeton et al. 1994). Alterations of the concentration of secreted proteins may be a way to regulate the symbiosis morphogenesis by changing the chemical structure of cell wall polymers. As a consequence, the mechanical properties of the cell walls and extracellular matrix involved in the symbiotic interface may be strongly modified. A prerequisite for the ectomycorrhizal sheath development around the host root is the tight contact between neighbouring cells. Hence, structural cysteine-rich proteins, such as hydrophobins, and enzymes that can covalently cross-link polysaccharides are candidates for extracellular proteins with a promotive role in morphogenesis.

Molecular cloning of SR fungal genes

Molecular studies on the *Eucalyptus globulus* – *Pisolithus tinctorius* interaction have demonstrated that the ectomycorrhizal fungus penetration down- or up-regulates transcript synthesis in the host root (Nehls et al. 1994). Also in the mycobiont, interaction with the host plant is accompanied by a shift in the expression of a set of developmentally related genes. Several clones were obtained by differential screening of a cDNA library of *E. globulus* – *P. tinctorius* mycorrhiza using cDNA probes prepared from (*i*) mRNA isolated from *P. tinctorius* colonized roots and (*ii*) mRNA isolated from free-living mycelium (Tagu et al. 1993). The SR transcripts represent about one third of the cDNA clones screened confirming early contentions (Hilbert et al. 1991; Martin and Hilbert 1991), based on protein analysis, that early developmental steps of ectomycorrhiza formation induce major shifts in fungal gene expression. According to protein synthesis data, it is known that mycorrhizal tissues are still expressing many of the genes that are expressed in free-living partners, but at a different level. This is confirmed by the analysis of mRNA populations and the drastic decreased expression of plant genes is especially noteworthy.

A detailed characterization of a dozen of these in planta regulated fungal genes is underway. For instance, a cDNA denoted *Mycf102'*, hybridized to a poly(A) mRNA of 1200 nucleotides. The level of *Mycf102'* mRNA is very high in the free-living mycelium, which presumably indicates a high rate of biosynthesis for the corresponding protein (Tagu et al.

Fig. 3. Comparison of the polypeptide sequence predicted by the cDNA EST141 from the ectomycorrhizal basidiomycete *Pisolithus tinctorius* 441 with known fungal hydrophobins. The predicted sequences of EST141 was aligned with the known hydrophobins. SC1, SC3, and SC4 are proteins from *Schizophyllum commune* (Wessels et al. 1991); Eas, RodA, and SsgA are translated from genes of *Aspergillus nidulans, Neurospora crassa* (Bell-Pedersen et al. 1992), and *Metarhizium anisopliae* (St. Leger et al. 1992); cerato-ulmin (C.U.) is a toxin peptide from *Ophiostoma ulmi* (Stringer and Timberlake 1993); Cryparin (Crp) is a peptide from *Cryphonectria parasitica* (Zhang et al. 1994). The sequences were aligned, as proposed by Stringer and Timberlake (1993), based on the conserved cysteine residues (shown in bold) using the SeqApp programme. Highly conserved cysteine motif is bolded (D. Tagu and F. Martin, unpublished data).

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                                  1
EST141                            LNARGGTP-- ---------- ----------
  Sc1    MPFS------ -LAILALPVL AAATAVP--- R--------- G---------
  Sc3    M-FARLPVVF LYAFVAFGAL VAALPGGHPG TTYPPSTTTI A---------
  Sc4    MRFS------ -LALLALPAL AAAAPVPGGG K--------- G---------
 RodA    MKFSIA---- AAVVAFAASV AALPPAHDSQ FAGNGVGNK- GNSNVKFPVP
  Eas    MQFTSV---- -FTILAIAMT AAAAPAEVVP R--------- ----------
  Mpg1   M-FSLK---- -TVVLAL--- AAAAFVQAIP AP-------- GE--------
  SsgA   M-FK------ -ALIVALAAV AAAIPTQQPS ---------- S---------
  C.U.   MQFSIA---- -TIALFSSAM AAPYS----- ---------- GNSN------
  Crp    MQFSII---- -AISFLASLA MASPAKRGGG GGGSGSGSGS GSGSGSGGG-
  Cons   M-FS------ ----LAL-A- AAA-P----- ---------- G---------

                10             20             30             40
EST141  --------SQ QCNT--GT-L QCCQQ----- -VQQTSDLQQ FLSSFG-LVD
  Sc1   -------GAS KCNS--GP-V QCCNT----- -LVDTKDKHQ TNIVGALLGL
  Sc3   -------AGG TCTT--GS-L SCCNQ----- -VQSASSSPV TALLG-LLGI
  Sc4   -------AGQ ACNS--GP-V QCCNE----- -TTTVANAQK QGLLGGLLGV
 RodA   ENVTVKQASD KCGD--QAQL SCCNKATYAG DTTTVDEGLL SGALSGLIGA
  Eas   ---ATTIGPN TC-SIDDYKP YCCQS----- ----MSGPAG SPGLLNLIPV
  Mpg1  -GPSVSMAQQ KCGA--EKVV SCCNS----- ---------- ----KELKNS
  SsgA  -------NEM NCDS--G--V YCCNK----- -V-----AQN TGIVVPIDAL
  C.U.  -----SDSYD PCTGLLQKSP QCCNT----- ---------- ----------
  Crp   -----STTYT ACSSTLYSEA QCCAT----- ---------- ----------
  Cons  -------A-- -C-S--G--V QCCN------ ---------- ---L--LLG-

               50             60         70
EST141  ALAGASALVG A--NCN---- -PVSVLGTGN -GAQCNTQPV ----CCTS--
  Sc1   DLGSLTGLAG V--NCS---- -PVSVIGV-G -GNSCSTQTV ----CCEG--
  Sc3   VLSDLNVLVG I--SCS---- -PLTVIGV-G -GSGCSAQTV ----CCEN--
  Sc4   VVGPITGLVG L--NCS---- -PISVVGVLT -GNSCTAQTV ----CCDH--
 RodA   GSGAEGL--G LFDQCSKLDV AVLIGIQDL- VNQKCKQNIA ----CCQNSP
  Eas   DLSA------ -SLGC----- ------VVGV IGSQCGASV- ---KCCKDDV
  Mpg1  KSGAEIPIDV LAGECKNIPI NILTINQLIP INNFCSDTVS ----CC----
  SsgA  ---------S S--TCG---- DTLKLVTVDA LNDKCTSQTV ----CCNN--
  C.U.  -----DILGV ANLDCHGPPS VPTSPSQ--- FQASCVADGG RSARCC----
  Crp   -----DVLGV ADLDCETVPE TPTSASS--- FESICATSG RDAKCC----
  Cons  -LG----L-G ----CS---- -PLSV-GV-- -G--C-AQTV ----CC----

          80          90   94
EST141  ---NQMLGAV NMG-CM-PLN VNA
  Sc1   ---TQFNGLV NVG-CT-PIN VGL
  Sc3   ---TQFNGLI NIG-CT-PIN IL-
  Sc4   ---VTQNGLV NVG-CT-PIS L--
 RodA   SSADGNLIGV GLP-CV-ALG SIL
  Eas   TNTGNSFLII NAANCVA--- ---
  Mpg1  --SGEQIGLV NIQ-CT-PIL S--
  SsgA  ---VQQNGLV NVA-CT-PID V--
  C.U.  ---TLSLLGL ALV-CTDPVG I--
  Crp   ---TIPLLGQ ALL-CQDPVG L--
  Cons  ----Q-[T/N]GLV N-G-CT-PI- ---
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1993). In contrast, this transcript occurs at very low levels in symbiotic tissues. Nucleotide sequencing of *Mycf102'*-cDNA and a search for homology in databases did reveal a significant homology with the inducible acid phosphatase of *Aspergillus niger*.

Products of in planta induced genes may be necessary for development and maintenance of the symbiosis. Characterization of fungal genes of which the expression is specifically induced or repressed in planta may, therefore, lead to the identification of so far unknown symbiosis factors.

Hydrophobins in ectomycorrhizal fungi

A recurring theme of recent findings in developmental biology in animals and plants is that the same set of genes is used to execute the same type of operation in multiple developmental processes. It is therefore possible that ectomycorrhiza development is a slightly modified version of other developmental programmes, such as fruiting body and sclerotium formation (Harley and Smith 1983). This is clearly illustrated by the expression of hydrophobin genes in *P. tinctorius* during ectomycorrhiza formation. Two transcripts, corresponding to the cDNAs EST32 and EST141, are preferentially expressed during the mantle formation. These transcripts are produced in the free-living mycelium and in planta. Normalization of the Northern hybridization signals suggested that these cDNAs represent mRNA species severalfold more abundant in planta than in free-living mycelium. The predicted amino acid sequences of these SR genes appear to have characteristics of a class of proteins known as the hydrophobins (Wessels 1992, 1993). Hydrophobins have been identified in a number of saprophytic and pathogenic fungi (Wessels 1993). *Pisolithus tinctorius* hydrophobin-like proteins (EST32 and EST141), like their *Schizophyllum commune* homologues, are small cysteine-rich proteins (90 and 150 amino acids) containing eight cysteine residues and presenting strongly hydrophobic domains (Fig. 3) (D. Tagu, B. Nasse, and F. Martin, unpublished data).

The amino acid sequence homology between hydrophobins of different fungal species is weak, and alignment relies heavily on the pattern of cysteine residues in the sequence (Stringer and Timberlake 1993) (Fig. 3). Another common feature is that the second and third cysteines form a doublet and are usually followed by an asparagine residue. Hydrophobin genes of *Neurospora crassa* (*eas*; Bell-Pedersen et al. 1992), *Aspergillus nidulans* (*rodA*; Stringer et al. 1991), and *Schizophyllum commune* (*Sc1, Sc2, Sc3*; Wessels et al. 1991; Wessels 1992) have been well characterized. Insoluble hydrophobins have been detected in the cell walls of several filamentous fungi (De Vries et al. 1993). The genes *eas, rodA*, and *Sc3* encode polypeptides that polymerize into insoluble rodlet arrays on the surface of conidia and aerial structure conferring hydrophobic properties to the hyphal surface (Stringer et al. 1991; Wösten et al. 1993). The less hydrophobic SC1 and SC4 are morphogenetic proteins that allow, or cause, hyphae to emerge off the substrate and to adhere to each other during development of aerial multicellular reproductive structures (e.g., conidiophores, basidiocarps) in filamentous fungi (Wessels et al. 1991; Stringer et al. 1991). They possibly play this role via intermolecular hydrophobic interactions and formation of disulfide bridges between hyphal surfaces (Wessels 1992, 1993).

The accumulation of hydrophobins onto the surface of the mycelium from pathogenic fungi suggests that they can play a role in cell-to-cell communication during the early stages of the interaction (Templeton et al. 1994). Disruption of the hydrophobin gene of the rice pathogen *Magnaporthe grisea* has shown that hydrophobin secretion is a key virulence factor, likely due to involvement of the hydrophobic protein in the formation of the appressorium (Talbot et al. 1993). The entomopathogenic fungus *Metarhizium anisopliae* also produces a hydrophobin, SSGA, which is expressed during appressorial formation and may be involved in attachment to hydrophobic surfaces (St. Leger et al. 1992).

Hydrophobins secreted by *P. tinctorius* and accumulating in inoculated roots might be involved in the aggregation of fungal hyphae ensheathing the root. In addition ectomycorrhizal hydrophobins produced during early stages of mycorrhiza formation could be secreted by colonizing hyphae onto the plant surface and possibly play a role in fungal attachment as suggested for another plant–microbe interaction (Talbot et al. 1993).

Concluding remarks and future research

During the development of ectomycorrhizas, a range of fungal tissues differentiates that can be distinguished by a combination of anatomical and cytological features (e.g., mantle, Hartig net). Fungal proteins and genes have been identified that are developmentally regulated during the differentiation of these symbiotic structures and whose expression correlates with changes in fungal and morphogenesis. Several fungal genes highly expressed in symbiotic tissues (e.g., SRAPs, hydrophobins) appear to have a structural function. These genes presumably play a specific and significant role in the development of symbiotic structures. The elucidation of the function of hydrophobin proteins and the regulation of hydrophobin genes will contribute to the understanding of the molecular mechanisms resulting in the formation of ectomycorrhizas. A comparative study of gene expression in different types of ectomycorrhizas might reveal to what extent similarities and differences in the various types of ectomycorrhizas are the result of variation in the basic mechanisms underlying the respective developmental programmes.

The penetration and colonization of the root tissues induce a drastic downregulation of the expression of plant transcripts and proteins. The mycobiont presumably optimizes the functioning of its nursing structure by downregulating the expression of some or all genes not essential to the modified root tissues. It would be interesting to identify the signals used by the fungal partner to modulate the gene expression of its host.

Future research should be directed to (*i*) the purification and sequencing of SRAPs and other cell wall proteins (e.g., gpD_{95}) and (*ii*) further characterization of SR genes (hydrophobin genes) and the corresponding proteins, in an attempt to understand their specific functions. The analysis of the spatial and temporal expressions of SR genes by in situ hybridization might help in the delimitation of specific symbiotic tissues and their organization into a coherently functioning ectomycorrhiza.

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